

***Escherichia coli* exonuclease III enhances long PCR amplification of damaged DNA templates**

Bernard Fromenty*, Christine Demeilliers, Abdellah Mansouri and Dominique Pessayre

INSERM Unité 481 and Centre de Recherches sur les Hépatites Virales de l'Association Claude Bernard, Hôpital Beaujon, 92118 Clichy, France

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ABSTRACT

Recent development of the long PCR technology has provided an invaluable tool in many areas of molecular biology. However, long PCR amplification fails whenever the DNA template is imperfectly preserved. We report that *Escherichia coli* exonuclease III, a major repair enzyme in bacteria, strikingly improves the long PCR amplification of damaged DNA templates. *Escherichia coli* exonuclease III permitted or improved long PCR amplification with DNA samples submitted to different *in vitro* treatments known to induce DNA strand breaks and/or apurinic/aprimidinic (AP) sites, including high temperature (99°C), depurination at low pH and near-UV radiation. Exonuclease III also permitted or improved amplification with DNA samples that had been isolated several years ago by the phenol/chloroform method. Amelioration of long PCR amplification was achieved for PCR products ranging in size from 5 to 15.4 kb and with DNA target sequences located either within mitochondrial DNA or the nuclear genome. Exonuclease III increased the amplification of damaged templates using either rTth DNA polymerase alone or rTth plus Vent DNA polymerases or Taq plus Pwo DNA polymerases. However, exonuclease III could not improve PCR amplification from extensively damaged DNA samples. In conclusion, supplementation of long PCR mixes with *E.coli* exonuclease III may represent a major technical advance whenever DNA samples have been partly damaged during isolation or subsequent storage.

INTRODUCTION

Long PCR (i.e. amplification of DNA fragments of several thousand base pairs) is now widely used in numerous fundamental or applied scientific fields and represents an invaluable diagnostic tool in molecular medicine (1–5). In 1994, long PCR technology took advantage of a seminal breakthrough based on the concomitant use in the PCR reaction of two DNA polymerases, one of which had a 3'→5' exonuclease, or 'proofreading', activity (6,7). The proofreading activity appears to be of importance for long PCR, as nucleotide

misincorporation into the nascent DNA strand can prematurely terminate DNA synthesis (6,7).

Despite this major discovery, long PCR is not always successful. Among the factors that are critical for the success of long PCR is template integrity. This is a matter of major concern whenever the experimenter cannot ameliorate the quality of DNA samples, either because the DNA was already damaged prior to extraction or because it was isolated under suboptimal conditions. Lesions that are able to hamper or block progression of the DNA polymerases include strand breaks, apurinic/aprimidinic (AP) sites and some base lesions (6,8,9).

We have recently used long PCR to look for mitochondrial DNA (mtDNA) damage, such as DNA strand breaks and AP sites (5). Low levels of AP sites were assessed in this study by pretreating DNA samples with reagents able to cleave DNA at AP sites prior to the long PCR amplification. This treatment transforms AP sites, which may only partially block progress of the DNA polymerases, into DNA strand breaks, which constitute absolute blocks for the polymerases (5). In more recent studies, we tested the effects of *Escherichia coli* AP endonucleases. The present study reports the surprisingly beneficial effect of one of these AP endonucleases, namely exonuclease III, on long PCR amplification of damaged DNA templates. This discovery may have important applications, enabling the successful use of long PCR with DNA samples that are mildly or moderately damaged.

MATERIALS AND METHODS

Origin and conditions for use of *E.coli* exonuclease III

Most experiments used exonuclease III from New England Biolabs, but some were performed with exonuclease III from Life Technologies. Exonuclease III (25 or 50 U unless otherwise indicated) was added directly to the PCR mixture along with the DNA polymerases. In some experiments, exonuclease III was heated for 10 or 30 min at 99°C before addition to the PCR reaction.

Isolation of DNA

Total DNA was isolated with Genomic-tip 100/G columns (Qiagen, Hilden, Germany) as described previously (5) or by phenol/chloroform extraction. Qiagen anion exchange resin usually yields larger DNA fragments (50–100 kb) than does phenol/chloroform extraction (8). DNA samples extracted with

*To whom correspondence should be addressed. Tel: +33 1 40 87 59 18; Fax: +33 1 47 30 94 40; Email: fromenty@bichat.inserm.fr

Qiagen Genomic-tip 100/G columns are thus particularly suitable for long PCR (5,8). Qiagen columns were used to isolate total DNA from mouse liver or mtDNA from isolated rat liver mitochondria. DNA samples isolated by the phenol/chloroform method were prepared from mouse liver and human blood (10) and were stored at -20 or -80°C for several years. Rat DNA samples extracted by the phenol/chloroform method were freshly prepared for the present study. DNA concentrations were assessed from the absorption at 260 nm.

Preparation of damaged DNA samples

DNA samples isolated with Qiagen columns were submitted to diverse DNA-damaging treatments. To create AP sites (11), DNA samples were treated with a depurination buffer (100 mM NaCl, 10 mM sodium citrate, pH 5.0) at 70°C for 0, 20, 40 or 60 min (DNA preparations AP0, AP20, AP40 and AP60, respectively). The number of AP sites created increases linearly with time (11). Other DNA samples were submitted to high temperature (99°C) for 30, 60, 90, 120 or 150 s, which induces AP sites, DNA strand breaks and possibly other DNA lesions (6,12). In other experiments DNA samples were denatured at 99°C for 10, 15, 20 and 30 min. Finally, some DNA samples were exposed to UV radiation (365 nm), generated by a Rad-Free UV lamp (6 W; Schleicher & Schuell) for 4.5 or 6 h. To avoid evaporation, the DNA samples were kept on ice. Near UV (300–400 nm) radiation produces DNA strand breaks, DNA–protein crosslinks and possibly AP sites (13–15).

Long PCR experiments

Long PCR was usually performed with the GeneAmp XL PCR system (Perkin Elmer), which uses a mixture of rTth and Vent DNA polymerases. Unless otherwise indicated, these experiments were performed as described previously (5). Other experiments were done with the Expand™ Long Template PCR system (Roche Molecular Biochemicals), which uses a mixture of *Taq* and *Pwo* DNA polymerases. These experiments were carried out as described previously (2,4), except that purified bovine serum albumin was omitted from the PCR reactions. Buffer 3, containing detergents and 2.25 mM MgCl_2 , was always used in the experiments performed with the Expand™ Long Template PCR system. Finally, some long PCR experiments were also done with rTth DNA polymerase (Perkin Elmer) alone, using the same PCR reaction mixture as for the mixed rTth plus Vent DNA polymerases.

All PCR reactions were performed in a volume of 50 μl overlaid with mineral oil, using MicroAmp reaction tubes (Perkin Elmer) and a RoboCycler Gradient 96 PCR apparatus (Stratagene). This multiple block instrument was shown to provide optimal cycling conditions for long PCR amplification (6).

Four different long PCR protocols were used. Protocol 1a was employed to co-amplify a long and a short DNA fragment from the mouse mitochondrial genome during the same PCR reaction, as described previously (5). Forward primer A 5'-CGACAGCTAAGACCCAACTGGG-3' (nt 470–492 of the mouse mtDNA sequence) (16) and backward primer B 5'-CCCATTCTTCCCATTTCATTGGC-3' (nt 785–762) amplified a 316 bp mtDNA fragment, while forward primer C 5'-TAC-TAGTCCGCGAGCCTTCAAAGC-3' (nt 4964–4987) and backward primer D 5'-GGGTGATCTTTGTTTGGCGGT-3' (nt 13599–13579) amplified an 8636 bp mtDNA fragment.

This four-primer long PCR protocol was originally designed to assess the number of lesions able to block the progression of DNA polymerases on the 8636 bp mtDNA target sequence (5). Indeed, random DNA lesions are much more likely to hinder the progress of DNA polymerases and decrease PCR amplification on long DNA fragments than on short DNA fragments (5). In this technique, co-amplification of the long and the short mtDNA fragment without spurious PCR products is achieved thanks to an imbalance in the amount of primers (i.e. 14 pmol for primers A and B, amplifying the short fragment, compared to 40 pmol for primers C and D, amplifying the long PCR fragment).

Protocol 1b was as Protocol 1a except that primers A and B were omitted from the PCR reaction. In both Protocols 1a and 1b, the Mg^{2+} concentration was 1.75 mM and the thermocycler profile was initial denaturation at 95°C for 2 min, 26 cycles of 95°C for 45 s, 57°C for 45 s and 68°C for 7.5 min, with a final extension at 68°C for 7 min. When the Expand™ Long Template PCR system was used to amplify the 8636 bp mtDNA fragment, the extension step was 12 instead of 7.5 min.

In Protocol 1c, short PCR reactions were carried out with primers A and B only, in order to amplify the short 316 bp mtDNA fragment exclusively, using either rTth DNA polymerase alone or a mixture of rTth and Vent DNA polymerases. General conditions of the PCR reactions were the same as for Protocol 1a except for the amount of primers A and B (30–40 pmol instead of 14 pmol) and the duration of the extension step at 68°C (1 instead of 7.5 min). All of the short PCR experiments were carried out with exonuclease III that had been preheated for 10 min at 99°C before addition to the PCR reaction.

Protocol 2 was designed to amplify almost the entire rat mitochondrial genome. Forward primer 5'-CACGAT-AGCTAAGACCCAACTGG-3' (nt 469–492 of the rat mtDNA sequence) (17) and backward primer 5'-CACCACAGTTAT-GTTGGTCATGGG-3' (nt 15879–15856) were used to amplify a 15411 bp (i.e. 15.4 kb) mtDNA fragment. Unless otherwise indicated, the amount of primers was 20 pmol and the Mg^{2+} concentration was 1.5 mM. The thermocycler profile for Protocol 2 included initial denaturation at 95°C for 2 min, 26 cycles of 95°C for 45 s, 58°C for 45 s and 68°C for 20 min, with final extension at 68°C for 7 min.

Protocol 3 was used to amplify a 5 kb fragment from the human CYP2D6 nuclear gene, as described previously (3). The forward primer was 5'-CCAGAAGGCTTTGCAGGCTTCA-3' and the backward primer was 5'-ACTGAGCCCTGGGAG-GTAGGTA-3'. The amount of primers was 40 pmol and the Mg^{2+} concentration was usually 1.5 mM, except for some experiments performed with 1.0 mM Mg^{2+} . The thermocycler profile for Protocol 3 included initial denaturation at 95°C for 2 min, 35 cycles of 95°C for 45 s, 63°C for 45 s and 68°C for 7.5 min, with final extension at 68°C for 7 min. The thermocycling conditions were unchanged when the Expand™ Long Template PCR system was used instead of the GeneAmp XL PCR system.

PCR products (22 μl) were electrophoresed through agarose gels (0.7–1.2%) containing ethidium bromide. Photographs were taken under UV transillumination and scanned with a Sharp JX-330 image scanning unit with ImageMaster1D software (Pharmacia Biotech) as described previously (5).

Southern blot hybridization

To assess the extent of DNA degradation, mouse total DNA samples were heated at 99°C for 30–300 s and Southern blot hybridization was performed as described previously (5). Briefly, unheated and heated DNA samples (2–5 µg) were loaded on 0.7% agarose gels without ethidium bromide. DNA was electrophoresed overnight at 2.5 V/cm, transferred to a Hybond N⁺ nylon membrane and hybridized with a radiolabeled 8.6 kb mouse mtDNA probe synthesized by long PCR. The different forms of the mouse mitochondrial genome (i.e. supercoiled, circular and full-length linear) were quantitated by densitometry of autoradiographs as described previously (5). Membranes were stripped and rehybridized with a mouse C₀t-1 nuclear DNA (nDNA) probe (Life Technologies). The high molecular weight DNA detected with the mouse C₀t-1 nDNA probe was quantitated by densitometry of autoradiographs, as described previously (5).

DNA sequencing and RFLP analysis of PCR products

DNA sequencing of PCR products was carried out with the Big Dye Terminator System (Applied Biosystems), as described previously (18). The 8636 bp mtDNA fragment amplified with Protocol 1b was submitted to DNA sequencing with primers C or D or with forward and backward internal primers (nt 7241–7261 or nt 13179–13159, respectively, of the mouse mtDNA sequence).

For RFLP analysis, the 8636 bp PCR product was digested with the restriction enzyme *EcoRV* or *BspMI* and the products visualized in a 3% MetaPhore[®] agarose gel (FMC BioProducts). *EcoRV* and *BspMI*, which have restriction sites at positions nt 5367 and nt 12936 on the mouse mtDNA sequence, respectively (16), cleave the 8636 bp mtDNA long PCR product close to each end, thus generating short fragments of 404 and 664 bp, respectively, along with the associated larger fragments.

RESULTS

Exonuclease III favors long PCR amplification of heat-denatured DNA samples

Heat induces AP sites and DNA strand breaks (6,12). To assess the extent of heat-induced DNA damage, mouse liver total DNA was heated at 99°C for various lengths of time and Southern blots were performed, first with an mtDNA probe and then with an nDNA probe (C₀t-1 mouse DNA). Using unheated DNA as reference, quantitation of the main forms of the mitochondrial genome (i.e. supercoiled, circular and full-length linear) and of the high molecular weight nDNA allowed us to assess the loss of mtDNA or nDNA induced by heat. After 30, 60, 90 and 150 s heating, 11, 32, 64 and 96% of the mtDNA was lost, whereas the loss of high molecular weight nDNA was 23, 82, 95 and 99%, respectively (data not shown). The loss of the main mtDNA forms and of the high molecular weight nDNA was accompanied by the appearance of DNA smears indicating degradation into numerous smaller DNA fragments.

Heat-denatured DNA samples were therefore used to assess the effect of exonuclease III on the long PCR amplification of increasingly damaged DNA templates. Mouse liver DNA samples were heated at 99°C for 30–120 s and were then submitted to long PCR Protocol 1a to co-amplify a 8636 bp mtDNA fragment and a 316 bp mtDNA fragment. In the

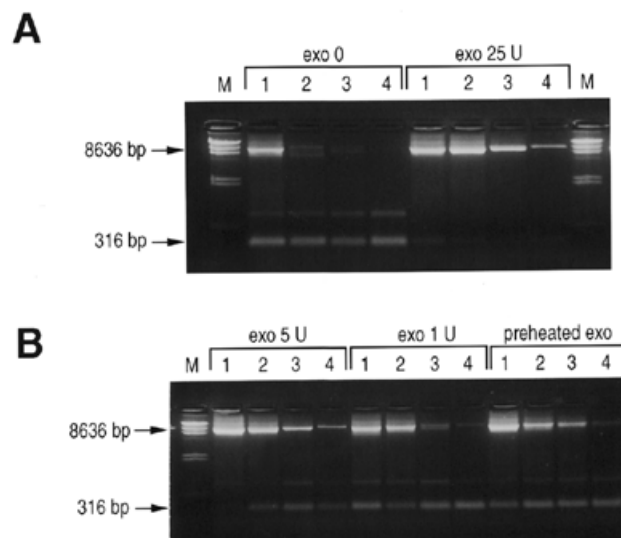


Figure 1. *Escherichia coli* exonuclease III enhances long PCR amplification of mtDNA from heat-damaged mouse liver DNA templates. Qiagen-extracted mouse liver DNA was heated at 99°C for 30–120 s and two distinct regions of the mtDNA were co-amplified with Protocol 1a using 14 pmol of primers for the 316 bp PCR product and 40 pmol for the 8636 bp PCR product. Lanes 1–4 correspond to aliquots of the same mouse liver DNA sample heated for 30, 60, 90 and 120 s, respectively. Agarose gels (1.2%) were loaded with 22 µl of the products. M is *HindIII*-digested phage λ DNA (fragment sizes 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb). (A) PCR reactions were performed without exonuclease III (exo 0) or with 25 U of exonuclease III (exo 25 U). (B) PCR reactions were performed with either 5 or 1 U of exonuclease III (exo 5 U and exo 1 U) or with 25 U of exonuclease III preheated at 99°C for 10 min (preheated exo).

absence of exonuclease III, the amount of amplified 8636 bp product decreased progressively with DNA templates heated for 30, 60, 90 or 120 s (Fig. 1A, exo 0). However, when exonuclease III (25 U) was added to the PCR mixture, there was a striking increase in the yield of the long PCR products (Fig. 1A, exo 25 U). A similar enhancing effect of exonuclease III was observed when the DNA polymerases were only added after the first DNA denaturation step (results not shown). Remarkably, exonuclease III restored significant amplification of the 8636 bp PCR product from the DNA sample heated at 99°C for 120 s, whereas this most severely damaged DNA sample was not detectably amplified without exonuclease III (Fig. 1A). However, whereas the yield of the long PCR fragment was enhanced by exonuclease III, we noted the disappearance of the short (316 bp) DNA fragment in the PCR product when 25 U of exonuclease III was used in the PCR experiments (Fig. 1A, exo 25 U). Since exonuclease III is able to remove mononucleotides from recessed 3'-termini, we surmise that high levels of the enzyme may have destroyed a significant amount of primers A and B (which are present in lower amounts than are primers C and D), thus explaining the preferential disappearance of the short PCR fragment.

We therefore attempted to reduce or inhibit the 3'→5' exonuclease activity of exonuclease III, either by decreasing the concentration of exonuclease III (19,20) or by preheating the enzyme (21,22) prior to the long PCR. Decreasing the concentration of exonuclease III to 5 U restored amplification

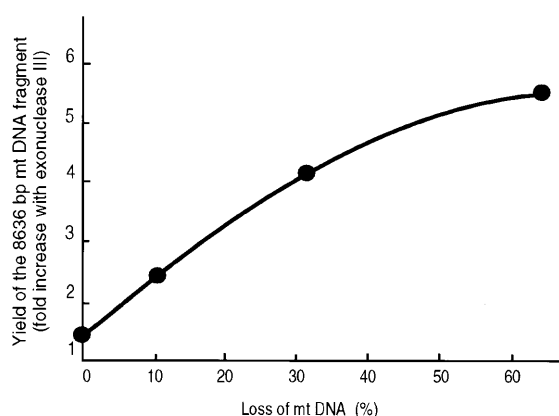


Figure 2. PCR product ratio (with/without exonuclease III) as a function of the percentage of heat-induced mtDNA loss. Four Qiagen-extracted mouse liver DNA samples were heated at 99°C for 0, 30, 60 and 90 s. Residual mtDNA was quantitated by Southern blot with an mtDNA probe while a 8636 bp mtDNA fragment was amplified with Protocol 1b, with or without 25 U of exonuclease III.

of the 316 bp DNA fragment, while fully maintaining its enhancing effect on the yield of the 8636 bp PCR product (Fig. 1B). However, the yield of the 8636 bp mtDNA fragment was slightly less with only 1 U of exonuclease III or with preheated exonuclease III (99°C for 10 min) (Fig. 1B).

Additional experiments with long PCR Protocols 1b, 2 and 3 confirmed that the amounts of primers in the long PCR reaction have to be sufficiently high to offset putative destruction of the primers by the 3'→5' exonuclease activity of exonuclease III (data not shown). In our hands, a minimum of 20 pmol of primers in the long PCR reaction seems to be required to take advantage of the beneficial effect of exonuclease III on long PCR amplification of damaged templates, but it might be necessary to adjust the primer concentration for other protocols.

In order to have a quantitative assessment of the beneficial effect of exonuclease III on long PCR amplification in relation to the severity of mtDNA damage, aliquots of several mouse liver DNA samples were heated at 99°C for 0, 30, 60 and 90 s and were used both to quantitate residual mtDNA by Southern blot hybridization and to amplify the 8636 bp mtDNA fragment using Protocol 1b (in the presence or absence of 25 U of exonuclease III). Exonuclease III increased the yield of the 8636 bp PCR product by 2.4-, 4.2- and 5.5-fold, while the percentage of mtDNA loss was 11, 32 and 64%, respectively (Fig. 2). Exonuclease III also increased the yield of the long PCR product from unheated mouse liver DNA samples by 1.4-fold on average, reflecting the unavoidable presence of some DNA damage, even in the most carefully prepared DNA samples (Fig. 2).

The much lesser enhancing effects of exonuclease III on the amplification of unheated DNA than heated DNA could have been due to a plateau effect in the PCR reaction. Hypothetically, a possible excess of undamaged DNA in the unheated sample could have hampered detection of a potential effect of exonuclease III in enhancing the amplification of undamaged DNA templates. To rule out this possibility, PCR amplification was compared with 100 (standard protocol), 10 and 1 ng of

unheated DNA. As expected, these dilutions progressively decreased the yield of the long PCR product. However, exonuclease III did not further increase the amplification of these diluted, unheated DNA samples (results not shown). By contrast, with heated 100 ng DNA samples (which similarly contain low amounts of residual undamaged DNA templates), exonuclease III markedly enhanced amplification, as already mentioned (Fig. 1). Thus exonuclease III has a preferential effect on damaged templates.

In order to assess the generality of these results, we first assessed the effect of exonuclease III on the amplification of an even longer mtDNA fragment, 15.4 kb in size, using Protocol 2 on heat-denatured rat liver mtDNA as the damaged template. We found that exonuclease III (25 U) significantly increased or allowed amplification of the 15.4 kb fragment for the mtDNA samples heated at 99°C for 30, 60 and 90 s (data not shown).

Structurally, mammalian mtDNA is atypical of most cellular DNA, as it is a small circular molecule. We thus wondered whether the beneficial effect of exonuclease III could also be observed for the long PCR amplification of damaged targets located within the nuclear genome. Using human blood DNA samples that were heat denatured for 30 or 60 s, exonuclease III (50 U) increased the yield of the 5 kb PCR product derived from the CYP2D6 human nuclear gene, but amplification was less frequently observed for DNA samples heated for 90 s (data not shown). This may be partly explained by the low number of copies of a given nuclear gene compared to a given mitochondrial gene in total DNA preparations, since there are hundreds or thousands of copies of mtDNA in a single cell, but only two copies of nDNA.

***Escherichia coli* exonuclease III favors long PCR amplification of depurinated DNA templates**

Since *E. coli* exonuclease III is an AP endonuclease (20,22), we assessed its ability to enhance long PCR amplification with depurinated mouse liver DNA samples. AP sites were created as described above and long PCR Protocol 1b was used to amplify the 8636 bp mtDNA fragment. Whereas in the absence of exonuclease III amplification progressively decreased with increasingly depurinated DNA samples, exonuclease III (25 U) restored normal amplification, even with the most depurinated DNA sample (AP 60) (Fig. 3). This beneficial effect was reproducible in seven series of experiments and, on average, exonuclease III increased the yield of the 8636 bp product with depurinated preparations AP0, AP20, AP40 and AP60 by 25, 30, 66 and 88%, respectively (data not shown).

To verify the nature of the 8636 bp PCR products amplified in the presence of exonuclease III, we sequenced some PCR products derived from the amplification of depurinated DNA samples (i.e. AP40). Our results indicated that the sequences of four different regions of these PCR products (representing a total of 2053 bp sequenced) matched the mouse mtDNA sequence reported by Bibb *et al.* (16). We also carried out restriction fragment length polymorphism analysis of the 8636 bp PCR products after amplification of depurinated DNA samples (AP40) with or without exonuclease III. Two restriction enzymes, namely *EcoRV* and *BspMI*, cleaved the 8636 bp PCR product close to each end, generating small DNA fragments of 404 and 664 bp, respectively. These end fragments were not detectably shortened by exonuclease III, indicating that its

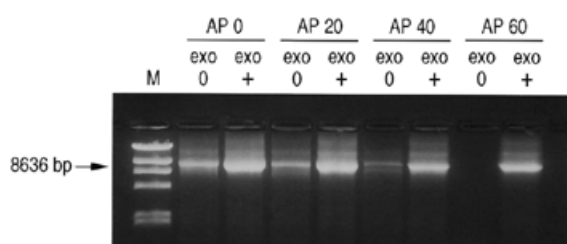


Figure 3. Exonuclease III enhances long PCR amplification of the 8636 bp mtDNA fragment from depurinated mouse liver DNA samples. Aliquots of the same Qiagen-extracted mouse liver DNA preparation were treated in depurination buffer at 70°C for 0, 20, 40 or 60 min (AP0, AP20, AP40 and AP60, respectively) and the 8636 bp mtDNA fragment was amplified with Protocol 1b without (exo 0) or with 25 U of exonuclease III (exo +). The agarose gel (0.8%) was loaded with 22 μ l of the PCR products. M, *Hind*III-digested phage λ DNA.

3'→5' exonuclease activity did not detectably trim the ends of the mtDNA-derived long PCR product (data not shown).

Exonuclease III augments long PCR amplification of phenol-extracted DNA samples

We also assessed whether exonuclease III would improve long PCR amplification with DNA templates that had been damaged during isolation and/or storage. We used mouse, rat and human DNA samples extracted with phenol/chloroform and either stored at –20 or –80°C for several years (mouse and human DNA) or recently prepared (rat DNA). Mouse liver DNA, rat liver DNA and human blood DNA were first subjected to Protocol 1a, Protocol 2 or Protocol 3, respectively, without exonuclease III and those DNA samples that gave little or no long PCR product were selected for further analysis.

As shown in Figure 4, exonuclease III increased amplification of the 8636 bp mtDNA fragment from mouse DNA samples (Fig. 4A), amplification of the 15.4 kb mtDNA fragment from rat DNA (Fig. 4B) and the 5 kb nuclear CYP2D6 product from human DNA (Fig. 4C). Interestingly, improved long PCR amplification of the latter product was also observed when the Mg^{2+} concentration was lowered from 1.5 (Fig. 4C) to 1.0 mM (data not shown), indicating that the beneficial effect of exonuclease III occurs over a wide range of Mg^{2+} concentrations.

Exonuclease III improves long PCR amplification of UV-irradiated DNA samples

Since exonuclease III may repair near-UV-induced DNA damage (14), we evaluated the effect of exonuclease III on long PCR amplification of the 8636 bp mtDNA fragment with mouse liver DNA samples submitted to near-UV radiation (365 nm). Again, exonuclease III increased the yield of the 8636 bp product; there was little or no amplification in its absence (data not shown).

Exonuclease III also improves long PCR amplification with rTth polymerase as the only DNA polymerase

The GeneAmp XL PCR system (Perkin Elmer) that was used in previously described experiments is based on the combination of two DNA polymerases, namely rTth and Vent DNA polymerases. Vent DNA polymerase has a 3'→5' exonuclease

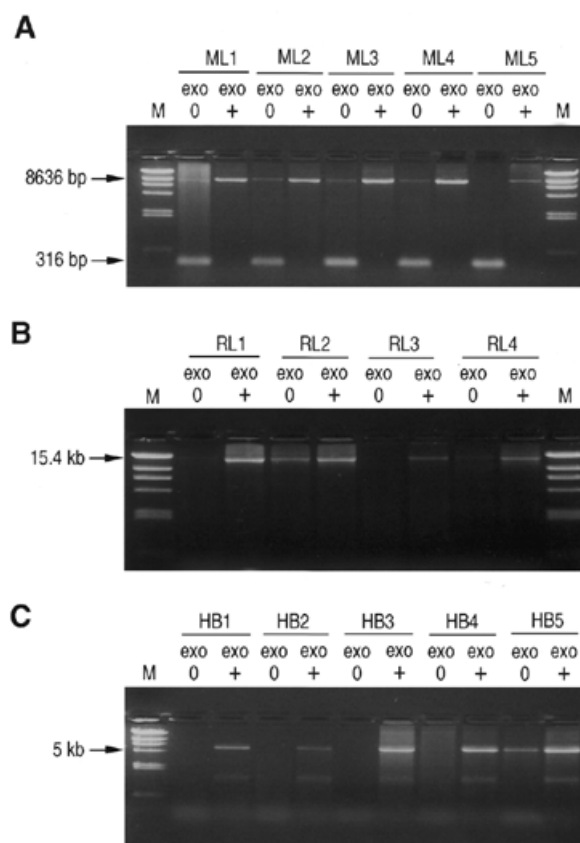


Figure 4. Exonuclease III enhances long PCR amplification from phenol-extracted DNA samples. DNA samples were extracted with phenol/chloroform and either stored at –20 or –80°C for several years (mouse and human DNA, respectively) or used immediately (rat DNA). After PCR, agarose gels (0.7–1.2%) were loaded with 22 μ l of the PCR products together with *Hind*III-digested phage λ DNA (M). (A) Five mouse liver DNA samples (ML1–ML5) were used for PCR co-amplification of the 316 and 8636 bp mtDNA fragments, using Protocol 1a without (exo 0) or with 25 U of exonuclease III (exo +). (B) Four rat liver DNA samples (RL1–RL4) were used for long PCR amplification of a 15.4 kb mtDNA fragment, using Protocol 2 without (exo 0) or with 25 U of exonuclease III (exo +). (C) Five human blood DNA samples (HB1–HB5) were used for long PCR amplification of a 5 kb fragment from the human CYP2D6 nuclear gene, using Protocol 3 without (exo 0) or with 50 U of exonuclease III (exo +).

(‘proofreading’) activity that appears to be essential for efficient amplification of long DNA targets. We asked whether exonuclease III could replace Vent DNA polymerase in this system. Although amplification with rTth DNA polymerase and exonuclease III did indeed occur, exonuclease III was not as efficient as Vent DNA polymerase in increasing the yield of the 8636 bp mtDNA fragment from mouse liver DNA samples (Fig. 5). This yield increased in the order: rTth polymerase < rTth polymerase + exonuclease III < rTth polymerase + Vent polymerase < both DNA polymerases + exonuclease III (Fig. 5).

The beneficial effect of exonuclease III on long PCR is observed with another combination of DNA polymerases and is similar with exonuclease III from two different suppliers

Long PCR systems other than the GeneAmp XL PCR system (Perkin Elmer) are commercially available. We thus assessed

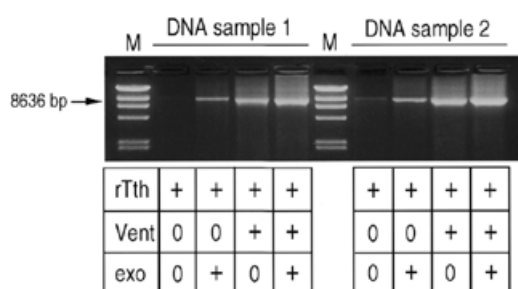


Figure 5. Effect of exonuclease III on long PCR amplification performed with either rTth DNA polymerase alone (rTth) or in combination with Vent DNA polymerase (Vent). Aliquots of two phenol-extracted mouse liver DNA samples were used for long PCR amplification of the 8636 bp mtDNA fragment in the absence (exo 0) or presence of 25 U of exonuclease III (exo +). The 0.8% agarose gel was loaded with 22 μ l of the PCR products. M, *Hind*III-digested phage λ DNA.

the effect of exonuclease III on long PCR amplification performed with the Expand™ Long Template PCR system (Roche Molecular Biochemicals), which combines the *Taq* and *Pwo* DNA polymerases. We found that exonuclease III enhanced the amplification of different types of damaged DNA templates when PCR reactions were performed with this combination of DNA polymerases (data not shown). Finally, the beneficial effect of exonuclease III on long PCR was similar with exonuclease III purchased from New England Biolabs or from Life Technologies (data not shown).

Exonuclease III does not afford a beneficial effect on short PCR amplification with extensively damaged DNA templates

Since exonuclease III exhibited a beneficial effect on long PCR amplification, we asked whether this enzyme might also have an effect on the amplification of short PCR fragments from extremely damaged DNA templates. It is noteworthy that in contrast to long PCR amplification, DNA samples had to be heat denatured (99°C) for at least 5 min in order to observe a decrease in amplification of the mtDNA-derived 316 bp PCR fragment (data not shown). This extended heat denaturation breaks DNA into tiny fragments and nucleotides.

We therefore used these extremely heat-damaged DNA samples to assess the effect of exonuclease III on amplification of the short 316 bp mtDNA PCR fragment. Preheated exonuclease III and high amounts of primers A and B (30–40 pmol) were used in order to limit the possible detrimental effect of the 3'→5' exonuclease activity on PCR amplification. No beneficial effect was observed when exonuclease III was used, either with rTth DNA polymerase alone or with the combination of rTth plus Vent DNA polymerases. Rather, a decrease in amplification of the short 316 bp mtDNA PCR fragment was often observed with these extensively denatured DNA samples (data not shown). Likewise, decreased amplification was observed with stored phenol-extracted mouse DNA samples that had yielded no 8636 bp mtDNA PCR fragment and little 316 bp PCR product (i.e. DNA samples that were undoubtedly severely damaged) in preliminary PCR experiments. Taken together, these data suggest that exonuclease III is unable to restore amplification of short PCR fragments from extensively degraded DNA templates.

DISCUSSION

We show here that *E.coli* exonuclease III strikingly improves long PCR amplification of damaged DNA templates. This beneficial effect seems to increase in proportion to the degree of DNA damage (Fig. 2 and Results), although it eventually disappears for extensively damaged templates. The beneficial effect of exonuclease III was observed both with DNA samples submitted to different treatments known to induce AP sites and/or DNA strand breaks (low pH, heat and near-UV radiation) and with phenol-extracted DNA samples stored for long periods. Exonuclease III increased long PCR amplification of DNA sequences of variable length (5–15.4 kb) located within the mitochondrial or nuclear genomes of different species. In addition, exonuclease III worked either with a single DNA polymerase (i.e. rTth DNA polymerase) or with different combinations of DNA polymerases (namely rTth plus Vent DNA polymerases or *Taq* plus *Pwo* DNA polymerases). It is also noteworthy that exonuclease III seems to be efficient within a broad range of Mg^{2+} concentration (i.e. 1.0–1.75 mM with the GeneAmp XL PCR system and 2.25 mM with the Expand™ Long Template PCR system). Taken together, these data suggest that exonuclease III may be effective under various conditions of long PCR amplification.

Among the numerous factors that are critical for long PCR amplification, integrity of DNA templates is of the utmost importance. When DNA has not been submitted *in vivo* or *in vitro* to DNA-damaging agents, lesions that are able to hamper or block progress of DNA polymerases during long PCR reactions are DNA strand breaks and possibly AP sites and base lesions (6,8,9). Regarding AP sites, their ability to either hamper PCR amplification or to be bypassed by DNA polymerases seems to be dependent of the number of lesions on the DNA template (Fig. 3). DNA synthesis may be impaired either when the proportion of AP sites is high on the DNA template or when AP sites are located in a particular sequence context, an event whose frequency is expected to rise as the number of AP sites increases (23–25). Finally, it is conceivable that a significant number of AP sites may be cleaved during the PCR reaction itself, thus generating lesions that are absolute blocks for DNA polymerases. Indeed, AP sites are known to be prone to spontaneous cleavage through the β -elimination process, especially at high temperature, in the presence of Mg^{2+} and when the DNA is single stranded (26,27).

In addition to its 3'→5' exonuclease activity, exonuclease III is known to exhibit AP endonuclease, 3'-phosphatase and 3'-repair diesterase activities (20,22,28). The AP endonuclease activity of exonuclease III is responsible for hydrolysis of the phosphodiester bond 5' to an AP site (class II AP endonuclease activity), thus leaving an intact nucleotide with a 3'-hydroxyl group that can serve as a primer for DNA polymerase. This endonucleolytic activity also operates at sites containing base lesions, such as urea and various *O*-alkylhydroxylamine residues (29,30), or benzene-derived adducts (31). The 3'-repair diesterase activity of exonuclease III is involved in its ability to remove different 3' damaged residues that block DNA polymerases for DNA strand synthesis (19,28,32), including 3'-phosphates, deoxyribose 5-phosphate and 3'-phosphoglycolate esters that may be generated at the sites of DNA strand breaks (19,32). Finally, it has been shown that exonuclease III is able

to remove one, two or even three mispaired 3'-terminal nucleotides (33).

The present data do not provide definite answers on the mechanism(s) for the enhancing effect of exonuclease III on the long PCR amplification of damaged templates. A first possibility could be that its 3'→5' exonuclease activity adds further proofreading activity, thus further improving long PCR efficiency. However, this effect may not be the only mechanism. Although exonuclease III slightly increased the performance of rTth DNA polymerase in the amplification of undamaged DNA templates, it was much less efficient than Vent DNA polymerase (Fig. 5), indicating that exonuclease III cannot fully substitute for a polymerase with 3'→5' proofreading activity. Furthermore, exonuclease III still improved the amplification of damaged DNA templates under experimental conditions which are known to decrease its 3'→5' exonuclease activity (Fig. 1). A second possibility is that exonuclease III may generate free 3'-hydroxyl ends at the level of previous AP sites or other damage. This could then allow extension by the DNA polymerases so that complete repair between the primer annealing sites could perhaps occur before the first PCR denaturation. However, the enhancing effects of exonuclease III persisted when the DNA polymerases were only added after the first denaturation step. Alternatively, the short DNA strands with free 3'-hydroxyl termini (which could be generated by exonuclease III) could then serve as 'natural primers' during subsequent PCR reactions. Annealing of such 'natural primers' could be favoured by the extended annealing/extension step used in long PCR experiments. Additional experiments are needed to distinguish between these (and possibly other) potential mechanisms.

In contrast to the long PCR experiments performed with moderately damaged DNA, a series of experiments showed that exonuclease III was not able to increase the amplification of extensively damaged DNA samples, irrespective of the size of the target sequence. As the number of lesions present on a DNA template increases, the number of DNA fragments harboring the entire amplifiable target sequence probably decreases to the point where it does not allow expression of the beneficial effect of exonuclease III. Thus, a factor that appears essential for the favorable effect of exonuclease III on PCR amplification of a DNA target is the extent of DNA damage within this target, rather than its overall length. Although the present study has focused on DNA templates ranging from 5 to 15.4 kb, exonuclease III should also prove beneficial on DNA targets whose size is outside these limits, provided that the targets are not too damaged.

In conclusion, the present findings show that exonuclease III is helpful for PCR amplification of long DNA templates. As DNA damage can occur during the extraction procedure (particularly with the phenol/chloroform method, but also with the Qiagen columns) and also during freezing/thawing of DNA samples, exonuclease III supplementation may be used systematically in long PCR reactions, especially when very long DNA sequences (i.e. >10 kb) have to be amplified. Exonuclease III supplementation might also prove useful for the PCR amplification of archaeological or archival DNA

samples, provided that the DNA templates are not too severely damaged. Finally, the ratio of PCR products obtained with or without exonuclease III might be used as an indirect index of the number of oxidative DNA lesions that are induced *in vivo* or *in vitro* by a variety of DNA-damaging agents.

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